ESTABLISHMENT AND IMPACT OF *PSEUDOMONAS FLUORESCENS* GENETICALLY MODIFIED FOR LACTOSE UTILISATION AND KANAMYCIN RESISTANCE IN THE RHIZOSPHERE OF PEA.

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SUMMARY

The impact of a *Pseudomonas fluorescens* strain, genetically modified for kanamycin resistance and lactose utilisation (the GMM), could be enhanced by soil amendment with lactose and kanamycin. Lactose addition decreased the shoot to root ratio of pea, and both soil amendments increased the populations of total culturable bacteria and the inoculated GMM. Only kanamycin perturbed the bacterial community structure, causing a shift towards slower growing organisms. The community structure with the GMM inocula in the presence of kanamycin showed the only impact of the GMM compared to the wild type inocula. The shift towards K strategy (slower growing organisms), found in the other kanamycin-amended treatments, was reduced with the GMM inoculation. Lactose amendment increased the acid and alkaline phosphatase, the phosphodiesterase activity and the carbon cycle enzyme activities, whereas the kanamycin addition only affected the alkaline phosphatase and phosphodiesterase activities. None of the soil enzyme activities were affected by the GMM under any of the soil amendments.

INTRODUCTION

Many studies have used genetic inserts (marker genes) to study the ecology of released bacteria in the environment, especially into the rhizosphere of crop plants as an aid to the study of the ecology of released biocontrol agents. Examples include the work of De Leij *et al.*,(1994a and 1995a) in studies on the establishment, survival and dissemination of a *lacZY, xylE* and kan^{r} modified *Pseudomonas fluorescens* strain. Rattray *et al.*,(1993), looked at the colonisation of *lux* marked *Pseudomonas fluorescens* strain, under varying conditions of temperature and soil bulk density in different components of the plant soil ecosystem. They did not examine differences between the wild type and the genetically modified derivative, but they found that temperature affected the survival and bulk density affected the colonisation of the inoculum. Flemming *et al.*,(1994) studied the survival in oil-contaminated microcosms of a *lacZY, lux* and rif ^{r} marked biosurfactant producing *Pseudomonas auruginosa* strain. They found that growth of wild type and GMM were similar in liquid culture. The inoculum was recovered in much higher numbers in the contaminated soil compared with non-contaminated soil, but the study did not extend to a comparison of wild type and GMM in the soil ecosystem. However, these authors did not look at the effect of the marker genes *per se* on the organism itself or on the ecosystem as a whole with soil inoculation.

England *et al.*,(1993) found no differences in survival and soil respiratory activity with inoculation of a wild type and genetically modified *Pseudomonas aureofaciens*. They (England *et al.*, 1995) also found no differences in straw, cellulose and calico cloth decomposition along with soil carbon source utilisation, as measured by BIOLOG, in soil microcosms with the two inocula. Elliott *et al.* (1994) marked *Streptomyces* strains with

actinorhodin production and found no differences between the transformant and wild type parent in wheat and alfalfa root colonisation.

It is possible that the performance (survival and colonisation) of inoculated transformants as biocontrol agents could be enhanced by biasing the environment towards the inocula, for example by the addition of antibiotics or other selective agents. Substrate dependent biological containment systems have been developed and inserted into the genomes of released organisms (Jensen *et al.,* 1993). van Elsas *et al.,*(1994) found a selective disadvantage to modified *Ps. fluorescens* strains (Tn 5 derived *nptII* or *nptII-cryVB* genes) when coinoculated with the wild type (WT) into soil microcosms. The addition of kanamycin to the system did not enhance the competitiveness of the kanamycin resistant GMM in comparison to the WT parent, but they did find an effect upon rhizosphere colonisation. de Oliveira *et al.,*(1995) investigated the effects upon the population dynamics of a Tn5 mutated *Ps. fluorescens* of kanamycin and streptomycin additions to soil, only streptomycin caused a significant stimulatory effect. Colbert *et al.,*(1993) found enhanced metabolic activity and increased population densities of biocontrol strain of *Pseudomonas putida* modified to utilise salicylate, in comparison with the wild type, in soil amended with salicylate. Larger numbers of the GMM were also isolated from the rhizosphere of sugar beet when the soil was amended with salicylate.

However, neither of these studies investigated the subsequent effects upon the ecosystem and its function as a whole. A number of methods have been shown to be useful indicators of perturbations caused by microbial inoculation. These methods notably include, rhizosphere microbial population structure (De Leij *et al.,*1994b) and soil enzyme activities

(Mawdsley & Burns 1995 and Naseby & Lynch 1997a) which is a measure of perturbation in ecosystem function (Naseby and Lynch 1997b).

We investigated the effects of wild type and GMM (*lacZY* and kan^r) inoculation in the rhizosphere of pea with the addition of selective agents (lactose and kanamycin) for the GMM, on soil resident populations and soil enzyme activities.

MATERIALS AND METHODS.

Soil description

The soil used was a sandy loam of the Holiday Hills series, taken from Merrist Wood Agricultural College 5 miles South-east of Guildford, and had been under permanent pasture for at least 15 years. The pH of the soil was 5.36, the particle ratio was 10:9:81 clay: silt: sand respectively, and the organic content was 1.6% by weight.

Experimental systems

Coarsely sieved soil (250g) was placed in an experimental microcosm consisting of a rolled acetate overhead projector sheet to form a cylinder 210mm high, slotted between the top and base of plastic 90mm diameter Petri dishes creating a semi-enclosed system.

Bacterial strains and treatment

The strains used were *Pseudomonas fluorescens* strain SBW25, a wild type strain isolated from the phytosphere of sugar beet, and its genetically modified derivative (strain SBW25EeZY-6KX), containing the marker genes *lacZY* (lactose utilisation), kanamycin resistance and *xylE* (catechol degradation), (Bailey *et al.*,1995).

The bacteria were grown on full strength tryptone soya agar for 4 days at 30^{o}C and each plate subsequently harvested in 10ml of sterile quarter strength Ringers solution using disposable plastic plate spreaders to scrape off the bacterial mat. Control plates (without

bacteria) were also flooded with quarter strength ringer's and surface scraped with spreaders. The resulting suspensions were collected in 250 ml Duran bottles and shaken vigorously to homogenise the bacterial suspensions. The bacterial suspension (containing $6x10^9$ ml^{-1} was subsequently used to imbibe pea seeds (var. Montana), at a ratio of one seed per ml, for 8 hours (stirred every 30 minutes) resulting in between 2 and 4 x 10^8 cfu per pea seed.

Experimental design

Five replicates of each of nine treatments containing eight imbibed seeds per microcosm, planted at a depth of approximately 1 cm below the soil surface were conducted. The treatments consisted of a control and seeds inoculated with wild type or GMM *Ps. fluorescens* in combination with kanamycin addition, lactose addition or no selective amendment to the soil. Soil amendments were added to each microcosm as a solution at rates 30ml of 1000ppm kanamycin or 30ml of 2% w/v lactose, in place of water, after seven days growth. The microcosms were placed in a growth chamber (Vindon Scientific) set to a 16 hour photoperiod with a day/night temperature regime of $21^oC/15^oC$ respectively, relative humidity was maintained at 70%.

Sampling and analysis

The plants were grown for 21 days before they were harvested, upon which soil closely associated with the plant roots was collected, sieved with a 2mm sieve, stored over-night at 4^oC and assayed for soil acid and alkaline phosphatase, phosphodiesterase, aryl sulphatase,

β glucosidase, acid β galactosidase and N-acetyl glucosaminidase by the methods of Naseby and Lynch (1997b).

Shoot and root fresh weights (after rinsing and drying) were measured and 1g of root was subsequently macerated and ground in 9 ml of sterile quarter strength Ringer's solution using an alcohol sterilised pestle and mortar. A ten fold dilution series was performed and P1 medium (Katoh and Itoh, 1983) amended with 50ppm Xgal was used for enumeration of the genetically modified, introduced strains. These plates were incubated at 25^{o}C and enumerated after five days growth. 10% tryptone soya agar was used for the enumeration of total culturable bacterial populations and for determining the population structure in terms of r and K strategists (De Leij *et al.*,1993) for this purpose the plates were incubated at 25^{o}C for seven days (counted on a daily basis).

Statistical analysis

Data was analysed using SPSS for Windows (SPSS inc.) by means of a one way ANOVA and subsequently differences between treatments (multiple comparisons) were elucidated by least significant difference.

RESULTS

Shoot/root ratio

The effects of soil amendment and microbial inoculation upon shoot, root and total plant weights (data not shown) individually were insignificant, therefore the data were transformed into shoot/root ratio (Clark and Reinhard, 1991). The addition of lactose significantly ($p<0.001$) reduced this ratio, in comparison to the control and kanamycin treatments, by approximately 15% (Fig 1). Kanamycin addition and both the microbial inocula (in non amended and both amended soils) had no effect upon the shoot/root ratio.

Bacterial populations and community structure

Lactose and kanamycin additions to the soil caused similar, significant increases in the total number of culturable bacteria in comparison with the non amended soil (Table 1). The two inoculated bacterial strains did not have a significant effect upon bacterial numbers in the non amended and lactose treatments. However with the addition of kanamycin, the control and WT inoculated treatments had much greater bacterial numbers than the GMM strain which had a similar population to the non amended treatments. The addition of kanamycin to soil did not effect the number of genetically modified *Ps. fluorescens* recovered (Table 2). However, lactose amendment significantly increased the number recovered by over 0.3 of a log unit.

Bacterial community structure measures the percentage of the total bacterial colonies appearing on inoculated agar plates over time. No notable differences were found in the

found in non-amended soil between the control and the two bacterial inocula, which peaked at 50h consisting of between 35 and 40% of colonies (Fig 2a). The addition of lactose to soil did not cause notable differences in the community structure profiles between the non inoculated and both inoculated treatments or the non amended treatments (Fig 2b). However, the addition of kanamycin to soil caused a number of changes to the community structure profiles (Fig 2c). The non-inoculated and WT inoculated had similar initial colony appearances (at 36h) to the non amended treatments. However, the colonisation patterns had a much smaller, broader peak (covering 50h and 74h), than their non-kanamycin amended counterparts, of between 25 and 30% of colonies. After 122 h the colony formation profile was similar to the control. The inoculation of the GMM in the presence of kanamycin resulted in colony formation baring a profile intermediate between the other kanamycin addition curves and the non kanamycin treatments. The initial colony appearance (36h) was much higher than the other kanamycin treatments and a narrow peak consisting of 35% of the colonies occurred at 50h, which was greater than peaks found in other kanamycin treatments but smaller than non kanamycin treatments. The peak rapidly declined to 15% at 74h and this percentage was maintained until 98h. After 98h the profile was similar to the other treatments.

Soil enzyme activities

Acid phosphatase activity (Table 3)was significantly greater ($p<0.001$) than the control with the addition of lactose. Alkaline phosphatase activity (Table 3) was double ($p<0.001$) and phosphodiesterase ($p<0.001$) activity (Table 3) was triple the control activities with the addition of lactose. Both activities were also significantly greater ($p<0.01$) than the activities in kanamycin-amended soil, which in turn caused greater activities than the

control ($p<0.05$). Lactose addition also caused a significant increase in the sulphatase activity ($p<0.001$) (Table 3). The bacterial inocula did not significantly affect the alkaline phosphatase and phosphodiesterase activities under any of the soil treatments.

Lactose amendment, as with the P cycle enzymes, significantly increased the acid and alkaline β galactosidase ($p<0.001$), the β glucosidase ($p<0.001$) and NAGase ($p<0.01$) activities (Table 4). However, effects of kanamycin amendment were insignificant.

DISCUSSION

The reduction in the shoot/root ratio with the addition of lactose may be an indication of plant stress, as this parameter is commonly used as an indicator of change in plant physiology (Clark and Reinhard, 1991). Causes of plant stress include pathogens and nutrient limitations (including oxygen, Drew and Lynch, 1980) and therefore a decrease in the shoot/root ratio may indicate such stress. The addition of simple sugars has been shown to increase the soil microbial biomass which reduces the available nutrients to the plant as they are locked up in the microbial biomass; Gallardo and Schlesinger (1995) found an increase in biomass N and a decrease in extractable N and P with the addition of dextrose to the soil. Wu *et al.*,(1995) found that native soil S was immobilised with the addition of glucose. Increased microbial biomass with the addition of carbon sources could also lead to the reduction in oxygen availability, this leads to anaerobic stress which can significantly affect plant growth (Drew and Lynch, 1980). The possibility that the plant was under increased nutrient stress is supported by the increase in acid phosphatase activity in the presence of lactose, phosphatase activity is inversely related to the available P (Tabatabai, 1982 and Tadano 1993). This indicates that P is less available to the plant as acid phosphatase is predominantly of plant (and fungal) origin (Tarafder and Marschner, 1994) and secretion has been shown to increase under P deficient conditions.

Both the lactose and the kanamycin additions increased the total culturable bacterial populations. The increase caused by the lactose amendment is likely to be a direct consequence of increased available carbon, creating a larger standing population, as was found by Gallardo and Schlesinger (1995) with an increased microbial biomass in dextrose amended soil. The mechanism by which the antibiotic amendment increased the bacterial

population may be different. Badalucco *et al.*,(1994) found that a large proportion of the indigenous soil microflora was eliminated by the addition of streptomycin and cyclohexamide, however, after two days the survivors used the dead portion of biomass as a nutrient source and some were also capable of using the antibiotic amendments as carbon sources. It is therefore possible that the kanamycin addition caused a similar effect, which was manifested as a larger culturable population, caused by a larger or more active (therefore culturable) population.

Lactose amendment did not cause a perturbation in the bacterial community structure. The simple carbohydrate addition is likely to be utilised primarily by r strategists (fast growing bacteria), which would give a large early peak in the bacterial community profile. However, this would not differ from the control profiles as young roots are predominantly populated by r strategists (De Leij *et al.*,1995b) and the experiment was sampled after only 3 weeks of plant growth. The addition of kanamycin caused the only significant effects upon the bacterial community structure profile. A large proportion of colonies appeared later in the control and *Ps. fluorescens* WT inoculated treatment than the non-kanamycin-amended counterparts. This suggests a shift towards slower growing bacteria (K strategists), caused by the antibiotic addition. This may be interpreted as a stress response, i.e. the kanamycin caused a level of stress to the indigenous community, resulting in a shift towards K strategy. However, the GMM inocula (kanamycin resistant) reduced the shift towards K strategy caused by the kanamycin amendment even though the kanamycin addition did not significantly effect the size of the introduced population. This may be a direct consequence of the community containing a large inoculated population of fast growing kanamycin resistant bacteria. This is a direct attribute of the genetically modified strain as the wild type inocula produced a similar profile to the control, where a large

proportion of fast-growing bacteria were killed off by the kanamycin amendment, leaving a wider niche for K strategists.

There are two possible mechanisms for the shift in colony-forming distribution, the first as proposed by Hattori (1982 & 1983) is a change in the physiological state of the bacterial cells. This theory suggests that starved cells (or more generally in this case, stressed cells) will take longer to form colonies than non-stressed cells. If it is assumed that the antibiotic addition caused a certain amount of stress in the indigenous bacterial community, then the shift towards later colony formation can be explained in terms of the physiological state of the bacterial cells. The alternative theory, as proposed by De Leij *et al.* (1993), is that there was a shift in the composition of the bacterial populations towards a slower growing community (towards K strategy). De Leij *et al.*, supported this theory by inoculating selected colonies isolated from soil onto fresh plates and recording the colony forming distribution again, finding a similar profile to the original isolation. However, this work used the inoculation of the same genetically modified organism as used in the present study but did not use the modifications in a functional sense as no substrate was added. The effect of functionally modified bacteria (especially modified in antibiotic resistance in the presence of the antibiotic) may be somewhat different. During this experiment a number of colonies from the kanamycin amended soil were re-inoculated onto fresh plates, and the colony formation was approximately equally distributed between colonies appearing after a similar time period to the initial soil isolates and others appearing 24 hours earlier than their respective parent colonies. Therefore it is likely that the mechanism for the distribution shift is some combination of the two theories.

Both the alkaline phosphatase and phosphodiesterase activities increased substantially with the addition to soil of lactose. This indicates (as with the acid phosphatase activity) that the lactose amendment caused a large demand for phosphate, with a greater carbon availability therefore causing phosphate to become more limiting. Similar results were found by Falih and Wainwright (1996) with the addition of sugar beet to soil as an easily available carbon (glucose) source. This amendment increased amylase, invertase, dehrdrogenase, urease, aryl sulphatase and phosphatase activities and also increased the total bacterial population. They concluded that the carbon input could be used to increase the rate of release of available ions from fertilisers such as insoluble phosphates. The release of such ions would be mediated by increased enzyme activities of microbial origin (as this experiment did not include a growing plant) released in response to increased C availability and a subsequent increase in P demand. Carbon cycle enzyme activities in the present study, increased with the addition of an easily available C source (as in Falih and Wainwright's work) as a direct consequence of increased carbon content and turnover.

The rapid proliferation of a small kanamycin-resistant community after the addition of kanamycin to soil would require rapid acquisition of nutrients, with phosphate being one of the most limiting, especially around a plant root. The highly active population (as seen with the high number of culturable bacteria) in a more P limited environment therefore led to a higher alkaline phosphatase and phosphodiesterase activity. Kanamycin amendment did not significantly affect the carbon cycle enzyme activities.

This study has clearly demonstrated the value and future potential of using genetically modified microorganisms in studying both biochemical processes and population dynamics in soil.

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Table 1. Total bacterial populations in the rhizosphere of pea as affected by lactose and kanamycin amendments.

	None			Kanamycin*			Lactose*		
Treatment#	Cont	WT	GMM	Cont	WT	GMM*	Cont	WT	GMM
Log cfu recovered	7.62 ±0.07	7.60 ±0.08	7.60 ±0.03	7.79 ±0.1	7.89 ±1.10	7.60 ±0.04	7.72 ±0.07	7.70 ±0..09	7.78 ±0.11

Treatments: Cont, non inoculated control; kan, kanamycin addition; lac, lactose addition; WT, inoculated with *Ps. fluorescens* SBW25 wild type; GMM, inoculated with genetically modified *Ps. fluorescens* SBW25.

*Significant effects, kanamycin addition, $p<0.05$; lactose addition, $p<0.05$; kanamycin addition with GMM inocula vs kanamycin addition with WT inocula, $p<0.05$.

Table 2. Recovery of genetically modified *Ps. fluorescens* from the rhizosphere of pea as affected by lactose and kanamycin amendments

Treatment#	GMM	Kan + GMM	Lac + GMM*
Log cfu recovered	5.954 ± 0.163	6.091± 0.12	6.294 ± 0.126

Treatments: kan, kanamycin addition; lac, lactose addition GMM, inoculated with genetically modified *Ps. fluorescens* SBW25.
*Significant effects, lactose addition, $p<0.05$.

Table 3. Phosphorus and sulphur cycle enzyme activities in the pea rhizosphere as affected by lactose and kanamycin amendments with the inoculation of wild type and genetically modified *P. fluorescens*.

	None			ΨKanamycin*			Lactose***		
Enzyme#	Cont	WT	GMM	Cont	WT	GMM	Cont	WT	GMM
Acid phos	2.57	2.43	2.46	2.49	3.02	2.68	3.12	3.42	3.08
Sulph	0.04	0.07	0.05	0.07	0.07	0.09	0.12	0.14	0.15
Alk phos	0.92	1.03	0.89	1.07	1.2	1.07	1.47	1.42	1.41
Diester	0.04	0.06	0.04	0.07	0.09	0.08	0.11	0.12	0.14

Activities expressed as mg pNP released/hour/g dry soil. Acid phos, acid phosphatase; Sulph, aryl sulphatase; Alk phos, alkaline phosphatase; Diester, phosphodiesterase Cont, non inoculated control; kan, kanamycin addition; lac, lactose addition; WT, inoculated with *P. fluorescens* SBW25 wild type; GMM, inoculated with genetically modified *P. fluorescens* SBW25.

*Significant effects, lactose addition, $p<0.001$. ΨAlkaline phosphatase and phosphodiesterase with kanamycin addition, $p<0.05$ and lactose addition vs. kanamycin addition, $p<0.01$

Table 4: Carbon cycle enzyme activities in the pea rhizosphere as affected by lactose and kanamycin amendments with the inoculation of wild type and genetically modified *Ps. fluorescens*.

	None			Kanamycin			Lactose**		
Enzyme#	Cont	WT	GMM	Cont	WT	GMM	Cont	WT	GMM
β gluc	0.38	0.39	0.38	0.39	0.43	0.49	0.80	0.84	0.76
NAGase	0.08	0.09	0.06	0.11	0.09	0.08	0.16	0.16	0.21
Acid gal	0.32	0.36	0.31	0.29	0.33	0.31	0.54	0.51	0.48
Alk gal	0.33	0.37	0.32	0.37	0.39	0.38	0.51	0.50	0.53

Activities expressed as mg pNP released/hour/g dry soil. Acid gal, acid β galactosidase; alk gal, alkaline β galactosidase; β gluc, β glucosidase; NAGase, N-acetylglucosaminidase. Cont, non inoculated control; WT, inoculated with *Ps. fluorescens* SBW25 wild type; GMM, inoculated with genetically modified *Ps. fluorescens* SBW25.
*Significant effects, lactose addition, $p<0.01$.

FIGURE LEGENDS

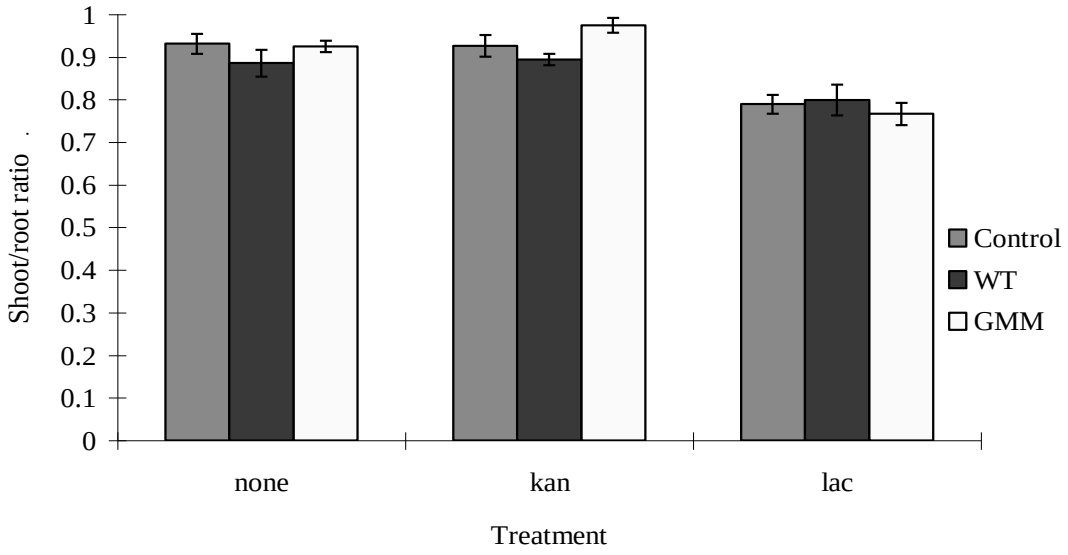
Figure 1: Ratio between plant shoot and root weights as affected by lactose and kanamycin amendments with the inoculation of wild type and genetically modified *Ps. fluorescens*.

None, no soil amendment; kan, kanamycin addition; lac, lactose addition; control, not inoculated; WT, inoculated with *Ps. fluorescens* SBW25 wild type; GMM, inoculated with genetically modified *Ps. fluorescens* SBW25.

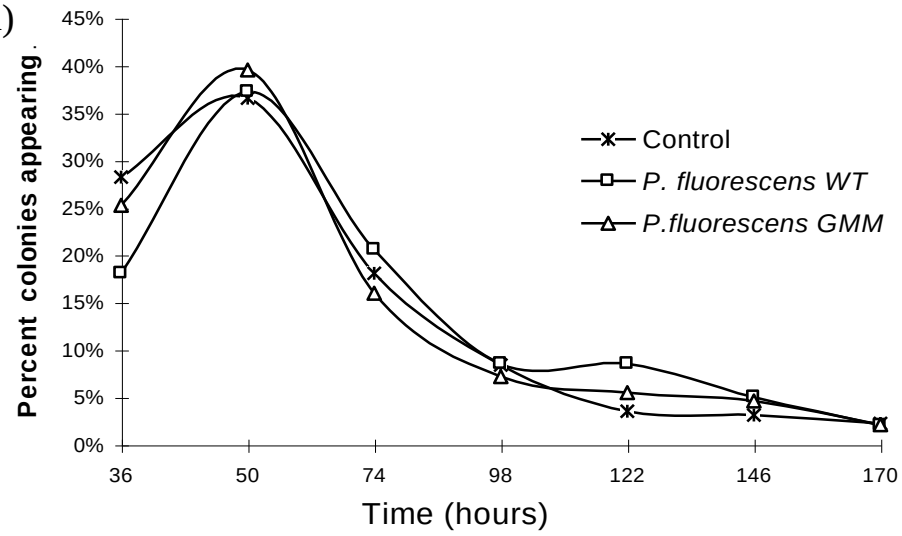
Standard errors of means shown (n=5). Significant effects, lactose addition, $p<0.001$.

Figure 2: Bacterial community structure in the pea rhizosphere with the inoculation of wild type and genetically modified *Ps. fluorescens* a) without soil amendment, b) with lactose amendment, c) with kanamycin amendment.

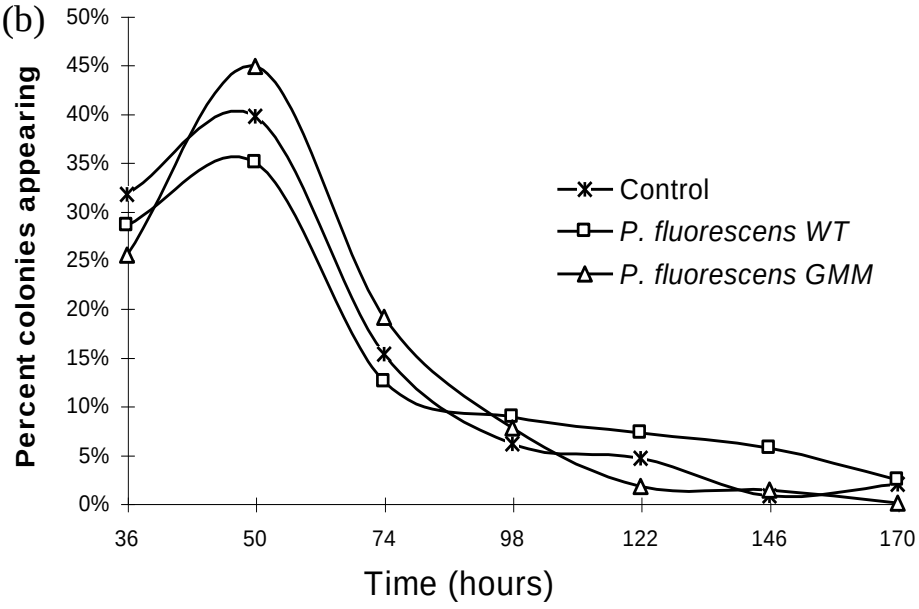
Control, not inoculated; WT, inoculated with *Ps. fluorescens* SBW25 wild type; GMM, inoculated with genetically modified *Ps. fluorescens* SBW25.


Shoot/root ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
none
kan
lac
Treatment
Control
WT
GMM

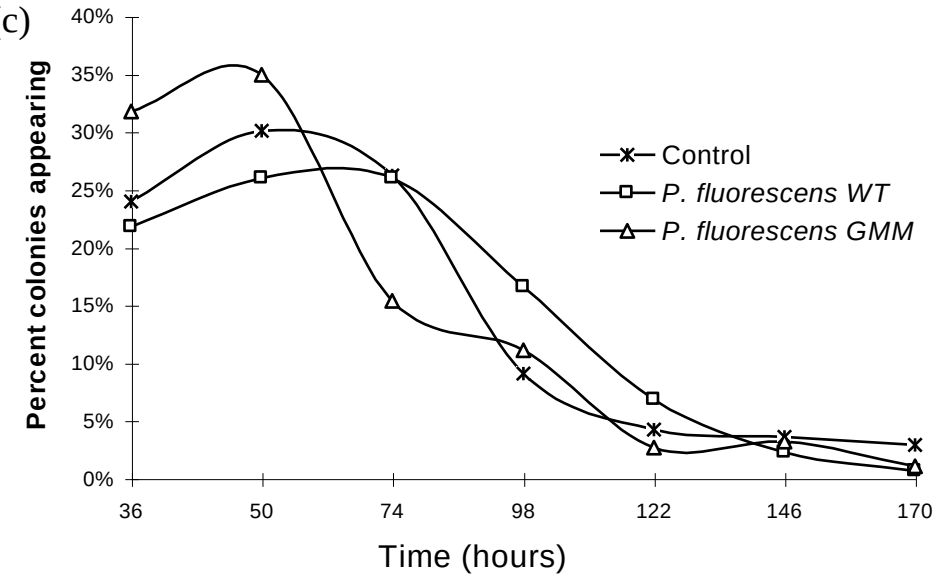
(a)


Percent colonies appearing
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
36
50
74
98
122
146
170
Time (hours)
Control
P. fluorescens WT
P.fluorescens GMM

(b)


Percent colonies appearing
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
36
50
74
98
122
146
170
Time (hours)
Control
P. fluorescens WT
P. fluorescens GMM

(c)


Percent colonies appearing
40%
35%
30%
25%
20%
15%
10%
5%
0%
36
50
74
98
122
146
170
Time (hours)
Control
P. fluorescens WT
P. fluorescens GMM